RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

Field of the Invention

The present invention generally relates to a recombinant potyvirus infectious nucleic acid construct useful for providing protection against viral infection in plants and to a recombinant virus harboring said construct. More specifically, the present invention relates to a recombinant potyvirus infectious construct containing an HC - Pro gene whose sequence coding for the conserved FRNK box contains a substitution. Preferably, the Arginin (Arg) is substituted with Isoleucine (Ile).

The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection.

Background of the Invention

The Curcurbitaceae is a broad botanical family comprising several economically important species cultivated worldwide, such as cucumber, squash, cantaloupe, zucchini pumpkin, melon and watermelon. Curcurbit production throughout the world is impaired by several aphid transmitted viruses, the most prevalent being the two potyviruses ZYMV (Zucchini Yellow Mosaic Virus) and WMV-2 (Watermelon Mosaic Virus 2) and CMV (cucumber Mosaic Virus). ZYMV infected plants show symptoms such as vein clearing followed by a yellow mosaic on the infected systemic leaf and may show stunting and distortion. In mild cases of infection the quantity and quality of the yield are damaged and in severe infections there might be a total loss of the yield, causing significant economical losses.

Control measures include phytosanitation, the use of colored plastic mulches for attracting virus bearing aphids and creating a hydrophobic barrier around the plant such as oil sprays. These provide temporary protection and are a limited protection during a massive infection.

Development of virus resistant cultivars either by classical breeding or by introducing viral derived nucleic acid sequences into the plant genome through genetic engineering of plants, is also employed for the protection of plants against virus infection. Squash hybrid transgenic inbred lines exhibiting resistance to ZYMV were produced (Tricoli D.M., Carney K.J., Russell McMaster P.F., Groff D.W., Hadden K.C., Himmel P.T., Hubbard J. P., Boeshore M.L. and Quemada H.D. (1995) *Biotechnology* vol. 13;1458) but these are limited to one cultivar only.

The phenomenon of cross protection, which is the use of a mild strain of a virus to protect against the damage by infection with severe strains of the same virus, provides a good method for controlling virus diseases.

In curcurbits, cross protection, specifically against ZYMV, is an attractive control option. Cross protection is highly effective under severe disease pressure. The severity of the disease conferred by the ZYMV on curcurbits and the latter's relatively short crop cycle (8 - 16 weeks) make cross protection a preferred control option for curcurbits.

The currently used mild strain of ZYMV for cross protection of curcurbits, was obtained by Lecoq (Lecoq H., Lemaire JM., Wipf-Scheible C., (1991) *Plant Dis.* 75:208-211). This strain is designated ZYMV-WK and is poorly transmitted by aphids, causes only mild leaf mottling and does not induce fruit malformation in curcurbits. Plants are inoculated at an early stage with the mild strain (ZYMV-WK), usually by mechanical inoculation.

No full length infectious clone of this mild virus exists.

Potyviruses have a genome consisting of a positive - sense single stranded RNA possessing a covalently linked 5' - terminal viral protein (Vpg) and a 3' terminal poly (A) tail. The viral RNA is expressed as a single polyprotein,

which is subsequently processed by three virus encoded proteases, producing eight to ten genes, which are a conserved region throughout the potyvirus genome. The potyviruses are transmitted from plant to plant by aphids in a non persistent manner, and this process is dependent on the presence of two virus encoded proteins, the coat protein (CP) and the helper component proteinase HC-Pro. The HC-Pro is a multifunctional protein involved in aphid transmission, polyprotein processing, virus replication, symptom expression and in virus movement in the plant (Maia I. G., Haenni A., and Bernardi F., (1996) *Journal of General Virology* 77:1335-1341).

Zucchini yellow mosaic virus (ZYMV) is a member of the potyvirus group which causes devastating epidemics in commercial curcurbits world wide. A full length clone of ZYMV, from which infectious transcripts were produced, was constructed (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643).

It was found that a substitution of the Alanin (Ala) residue to Threonin (Thr) at position 10 in the conserved DAG (Aspartate - Alanin - Glycine; Asp-Ala-Gly) triplet in the N terminal region of the CP effectively abolished aphid transmissibility of ZYMV (Gal On A., Antignus Y., Rosner A., and Raccah B. (1992) *Journal of General Virology* 73:2183-2187). Also substitution of Thr by Ala at position 309 in the HC-Pro gene of the infectious clone of ZYMV effected aphid transmissibility without changing virus accumulation and symptom development (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414), though less effectively than the substitution in the DAG triplet in the CP of the ZYMV.

It has surprisingly been found that an amino acid substitution in the conserved FRNK box of the potyvirus HC-pro gene allows for the construction of an infectious potyvirus construct, which, when introduced to plants, induces little or no symptom development, and which does not effect the accumulation of the virus in the plant. This infectious construct is therefore a unique potyvirus construct which is highly superior for plant cross protection and for transient

expression of foreign nucleic acid in plants. It has an improved ability of protection against infection by the severe strain of ZYMV, over any of the existing protection methods, is significantly safer and more environment friendly than the naturally occurring viruses, does not cause the development of symptoms in a variety of curcurbits, and is stable (no revertant virus has been found after several passages through plants).

Summary of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg. Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility, such as a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

The recombinant construct of the present invention may be useful for plant cross -protection (especially against severe strains of ZYMV) and for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone (defective RNA). The full length clone may be of any potyvirus, preferably of ZYMV.

The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

The present invention also relates to a method for introducing foreign nucleic acid into plants according comprising infecting a plant with a full length clone or co-infecting a plant with a full length clone, from which any viral genes are

removed, together with a full length clone or virus harboring a full length clone.

The present invention also relates to a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny, and to a virus produced in this method.

The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

Detailed Description of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid construct useful for plant cross protection and for the transient expression of foreign nucleic acid in plants. The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

The construct of the present invention comprises a full length potyvirus clone containing a substitution in the conserved FRNK box sequence in the HC - pro gene, preferably, Arg (in the FRNK box) is substituted with an amino acid having a bulky side chain or an amino acid from the hydrophobic group such as Ile. This substitution in the FRNK box dramatically effects the severity of symptom development without effecting the accumulation of the virus in the plant. Preferably, the construct of the present invention also contains a substitution which effectively abolishes aphid transmissibility , such as the substitution of the Ala residue to Thr at position 10 in the conserved DAG (Asp-Ala-Gly) triplet in the N terminal region of the CP or substitution of Thr by Ala at position 309 in the HC - pro of ZYMV.

Full length infectious clones of a severe strain of ZYMV were constructed and put under the control of a phage promoter, such as the T7 RNA polymerase promoter (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), bacterial promoters or a promoter effective *in planta*, such as the cauliflower mosaic virus (CaMV) 35S promoter (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

In the work presented here, the FRNK box is implicated, for the first time, as being of importance in symptom development, surprisingly without effecting the accumulation of the virus in the plant. Due to the highly conserved sequence of the FRNK box within the HC -Pro gene of the potyviruses, any substitution in the FRNK box of a potyvirus would have an effect on symptom development, not only the substitution of Arg in position 180 with Ile, in ZYMV, demonstrated in the work described here.

Based on the highly conserved genome, organization and gene function of the potyviruses, it may be concluded that the conserved FRNK box in the HC - pro gene has the same function in all potyviruses (perhaps as a receptor). Therefore, the substitution in the FRNK box in any of the potyviruses would have a similar effect on symptom development. Members of the potyviruses that are economically important are, for example, BCMV (Bean Common Mosaic Virus), BYMV (Bean Yellow Mosaic Virus), BtMV (Beet mosaic), MWMV (Moroccan watermelon mosaic), OYDV (Onion yellow dwarf), PRSV (Papaya ringspot), PStV (Peanut stripe), PepMoV (Pepper mottle), PVMV (pepper veinal mottle), CGVBV (Cowpea green vein banding), GEV (ground eyespot), ISMV (Iris severe mosaic), JGMV (Johnsongrass mosaic), LYSV (Leek yellow stripe), LMV (Lettuce mosaic), MDMV (Maize dwarf mosaic), PPV (Plum box), PVA (Potato A), PVV (Potato V), PVY (Potato Y), SbMV (Soybean mosaic), SCMV (Sugarcane mosaic), SPFMV (Sweet potato feathery mottle), TEV (Tobacco etch), TVMV (Tobacco vein mottling), TBV (Tulip

breaking), TuMV (Turnip mosaic), WMV-2 (Watermelon Mosaic Virus 2) , YMV (Yam mosaic), ZYFV (Zucchini yellow fleck).

The infectious clone may be an RNA transcript or a cDNA construct, though the use of infectious transcripts is the less efficient process *in vitro*.

A method for providing protection against viral infection in plants, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct, for example, by mechanical inoculation or by bombardment.

Compositions containing, as an active ingredient, the construct of the present invention may be used for superior plant cross protection, especially against infection by the severe strain of ZYMV and for transient expression of foreign nucleic acid in plants. The composition used for the introduction of the construct into plants, for infecting them by bombardment is an aqueous composition comprising, in aproximately equal volumes, the construct, a salt, such as calcium nitrate and particles such as tungsten, gold. The composition used for the introduction of the construct into plants by mechanical inoculation comprises infected plant tissue.

The construct of the present invention may be further used as a vehicle for the transient expression of foreign nucleic acid, namely genes, in a plant. The construct according to the present invention is highly infective, does not induce symptoms in the infected plants and is not transmitted by aphids.

Use of compositions, containing as an active ingredient, this clone provides an efficient, safe and environment friendly method for transient expression of foreign nucleic acid into the infected plants. Further applications of this construct may, therefore, be the expression of foreign sequences or genes within a defective RNA molecule of potyviruses. Defective RNAs are viral RNA genomes which are missing some of the viral genes but which, together with a complete helper virus (the full length parental virus), can facilitate the expression of the sequences they contain. Defective RNAs are derived from the helper virus genome, but still require the presence of a complete helper

virus for replication in the plant cell. The construct of the present invention may have viral genes removed from the full length clone and may then serve to support the expression of foreign genes via potyviruses defective RNA by co-infection of a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for introducing foreign nucleic acid into plants according to the present invention comprises infecting a plant with a full length clone into which any sequence of DNA or RNA is inserted or co-infecting a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for the production of a mild strain of potyvirus, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct and collecting the resulting progeny.

The said invention will be further described and illustrated by the following experiments and figure. These experiments and figure do not intend to limit the scope of the invention but to demonstrate and clarify it only.

Brief Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d).

Detailed Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d). The open and striped bars indicate the ZYMV-NAT and ZYMV-WK sequences within the FLC respectively. The relevant restriction enzymes and the amino acid changes are present. On the right side the severity of the symptoms in squash is indicated, from very severe (+++++) to mild (+). The sequence of the primer used for the mutagenesis is indicated.

Example 1 - full length clone (FLC) of ZYMV

Construction of the mutants in the full length clone (FLC) of ZYMV

The constructs which represent the HC - Pro sequences (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414) of the ZYMV - WK strain were placed under the T7 RNA promoter in the infectious FLC. In order to get higher rate of infection with those constructs the fragment BstXI/AgeI from the FLC of 35SZYMVNOS cDNA (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643 and Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227), was replaced by the appropriate fragment from pZYHC (-) clone (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414). Site directed mutagenesis was introduced on ssDNA template of the subclone pksM16B (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), using the primer 5' ATGTTCATAAATAAGCGCTCTAG3' (amino acid Ile is underlined and the unique restriction site of Eco47III is in bold). The clone pksM16B carrying the mutations was double digested by BamHI/BstEII and the obtained fragment (1.4kb) was introduced to the same sites in the 35SZYMVNOS cDNA (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

Plants, mechanical or bombardment inoculation and symptom appearance of the ZYMV AG1

Greenhouse - grown zucchini squash (*Curcurbita pepo*. L. cv Ma'ayan), cucumber (*Cucumis sativus* L. cv. Bet Alpha; Shimshon; Delila), melon (*Cucumis melo* L. cv. Arava) and watermelon (*Citrullus lanatus* Schad cv. Malali) plants were used at the cotyledon stage. The inoculated plants were maintained in a growth chamber under continuous light at about 25^{0}C. The plants were examined daily for visual symtom development.

Bombardment inoculation were as previously described by Gal - On et al. (1995). Mechanical inoculation of plants infected by the recombinant virus were performed by sap inoculation (100mg/ml), applied to a cotyledon previously dusted with carborundum.

Cross protection experiments

Cross protection by the ZYMV-AG1 strain was tested as described by Lecoq et al. (1991) Squash seedlings at the fully expanded cotyledon stage were bombarded with the 35S-AG1 at 0.1 μg/μl. A week later they were infected in the greenhouse by 5 - 7 aphids (*Myzus persicae*) per plant according to Antignus Y., Raccah B., Gal - On A. and Cohen S. (1989) *Phytoparasitica* 17:287-289).

Determination of the mutation in the progeny virions

To ascertain the presence of the mutations in the viral RNA total mRNA from infected leaf tissue was extracted. The synthesis of the RT-PCR was performed as described by Huet et al. (1994).

ELISA assay for evaluation of ZYMV titer

Leaf discs of squash and cucumber ZYMV-infected plants were taken 7 - 10 d.p.i. and the homogenized tissue were subjected to ELISA as described by Antignus et al (1989).

Previously, sequence comparison has shown four amino acid changes in the 455 amino acid sequence of the HC - pro gene between the severe field strain (ZYMV - JV) and the mild field strain ZYMK - WK. The replacement of a fragment of the HC - Pro of ZYMV - WK containing two substitutions Aspartate (Asp) 148 and Arg 180 (BstXI/BstEII fragment), reduced symptom expression of the virus in squash plants without effecting virus accumulation. To distinguish which of the two substitutions, Asp 148 or Arg 180, effect

symptom development, Arg 180 was replaced by Ile within the FRNK box (figure 1, clone d) by site directed mutagensis.

The engineered virus containing the Arg 180 replacement by Ile, was designated ZYMV-AG1. This new strain did not cause the development of symptoms in cucumber (three different varieties), melon and watermelon. The virus did accumulate to levels as high as that of the wild type ZYMV-JV. It was assumed, therefore, that the second amino acid difference (Asp at position 148) is dispensable for altering the symptoms from mild to severe.

In order to verify the presence of the amino acid changes within the mild virus ZYMV - AG1, and to prevent aphid transmission, a new restriction site of Eco47III was introduced at position 550 nt (from the 5' of the HC- Pro gene) and the DAG motif in the CP was replaced by DTG respectively (figure 1).

The new engineered virus (AG1) and a wild type severe strain (JV) accumulated to a similar level in systemically infected leaves of different curcurbit species (Table 1). Therefore, it may be concluded, that a point mutation changing amino acid Arg 180 to Ile, dramatically effects the severity of symtom development without effecting the movement and the replication of the ZYMV virus in the plant. The dramatic results confered by a point mutation in the potyvirus FRNK box, demonstrated in this work for the first time, could not have been inferred from the mere known sequence comparison which showed amino acid changes between the severe field strain and the mild field strain.

The stability of the amino acid substitution Arg 180 to Ile within ZYMV-AG1 was tested by infecting hundreds of squash plants and dozens of cucumber plants (Table 2). The presence of the Ile 180 mutation in the HC - Pro was confirmed by sequencing (data not shown). Curcurbit plants inoculated with ZYMV-AG1 mechanically or by particle bombardment with the ZYMV-AG1 strain did show the mild symptom appearance even throughout the growing period of the plant (Table 2). The presence of the Ile 180 mutation within the

virion genome was confirmed by sequencing or indirectly by digestion of the RT-PCR amplified fragment with the restriction enzyme Eco47III (figure 1). Replication and movement of the engineered ZYMV-AG1 strain remained high (as the wild type ZYMV), as seen from the accumulated level of the virus. These results suggest that no selective pressure is exerted to cause a reversion in the virus mutated genome.

The ability of the newly produced mild strain (ZYMV-AG1) to protect against a challenge inoculation of the severe strain of ZYMV (JV), was studied in cross protection experiments. Most of the protected plants did show mild symptoms after a challenge with the severe strain (96% protection). Two plants out of 47 that were infected with the ZYMV-AG1 strain and challenged a week later with the JV strain exhibited severe symptoms about one month post inoculation (Table 3).

The protection was studied in a small field experiment in which protected plants were exposed to field inoculation. Approximately 40% of the control non-protected plants became infected, while none of the protected plants showed severe symptoms. Therefore, no fruit damage was observed in the protected plants (Table 3). Previous studies showed that in a typical cross protection phenomenon, both the protective and the challenge virus strains are very closely related (Perring T.M., Farrar C. A., Blua M. J., Wang H.L. and Gonsalves D. (1995) *Crop Protection* 14 no. 7, 601 - 606). This is the first report where cross protection takes place between strains that have an identical sequence, including the coat protein sequence, that differ only in a single amino acid in a non structural protein (the HC - Pro).

2) Cross protection in melons

Melon (*Cucumis melo* L. cv. Ofir) seedlings were planted and were infected with ZYMV-WK and the recombinant virus ZYMV-AG1. The viruses were sprayed onto the melon seedlings prior to planting. The seedlings were then planted together with untreated (control) seedlings.

Half of the plants at three weeks were challenged mechanically with the wild type virus (ZYMV-JV) and half were unchallenged for testing natural infection.

30 days after the begining of the experiment parameters such as the plant size and the extent of infection with the wild type virus, were studied. Plants infected with ZYMV-JV that were not treated by the weakened virus (WK) were small and showed clear infection symptoms. Plants treated with the recombinant virus (ZYMV - AG1) showed no symptoms of infection.

3) Expression of foreign genes through the ZYMV-AG1 clone in plants

For the expression of a foriegn gene in an infected plant, a Pst I site was inserted into the ZYMV-AG1 between the NIb and CP genes. The GFP (green flurocent protein) reporter gene and the Bar gene, which confers resistance to the non selective herbicide bialaphos (commercially named BASTA), were amplified by PCR, using primers containing the Pst I restriction site, and were inserted in the PstI site.

Plants were inoculated by bombardment with the ZYMV - AG1 containing the GFP reporter gene or Bar gene.

Biochemical analysis showed the GFP and Bar gene to be highly and stably expressed. Even after several passages, no revertants of the recombinant mild virus were found and the reporter gene and Bar expression remained high and stable. Plants expressing the GFP were luminecent and plants expressing the Bar gene were found resistant to the herbicide bialaphos.

Table 1. Comparison of virus accumulation between ZYMV-JV and ZYMV-AG1 strains in cucurbits.

experiment no.	no of tested plants: JV, AGI, WK	ZYMV-JV# severe ELISA OD(405)	ZYMV-AG1^ mild	ZYMV-WK~ mild
1s+	11, 6, 6	0.9* (0.41)**	0.5 (0.19)	0.7 (0.18)
2s	2, 9, 8	1 (0.4)	0.7 (0.48)	-
3s	3, 10, 4	0.3 (0.08)	0.9 (0.27)	1.33 (0.13)
4s	9, 9, 9	0.51 (0.4)	0.46 (0.21)	0.59 (0.3)
5s	9, 9, -	0.56 (0.07)	0.7 (0.09)	-
6s	9, 8, -	0.82 (0.09)	0.95 (0.09)	-
7c	6, 7, -	0.7 (0.07)	0.81 (0.2)	-

Severe strain of ZYMV which found in Israel in the Jordan Valley (JV).

^ The engineered virus of ZYMV.

~ ZYMV weak strain described by Lecoq et al. (1991).

* Average of O.D (405) detected by ELISA from 11 plants.

** Standard deviation (in brackets).

+ s and c are squash and cucumber test plants, respectively.

Table 2. The stability of the ZYMV-AGI virus in the plants

plant species	number of tested plants: bombardment with 35SAG1	* visual symptoms: mild	* visual symptoms: severe	# molecular analysis of Ilu-180 mutation
squash	402	398	0	10
cucumber	105	103	0	5
melon	30	30	0	3
Total	537^	531+	0	18

*Visual symptoms were observed and detected by ELISA about one and half month post inoculation.

The presence of the Ilu Mutation was confirmed by digestion of the RT-PCR by Eco47III restriction enzyme.

^ Total of bombarded plants.

+ Total of infected plants

Table 3. Cross protection in squash with the mild strain ZYMV-AG1 (induction) against the severe strain ZYMV-JV (challenge) in the greenhouse experiments.

	Number of tested plants				
experiment number*	induction ZYMV-AG1	#challenge ZYMV-JV	symptoms mild	severe	~fruit damage
a)	47	47	45	2	1
a)	14	-	15		0
a)	-	5		5	5
b)	15	15	14	0	0
b)	5	-	5		0
b)	-	5		5	5
c)	43	field inocul.	43		0
c)	-	6		6	6
c)18 healthy	-	field inocul.		7	7

* a, b and c are three separate experiments. a and b were in the greenhouse and c was done in a small plot in the field. c is a sum of two experiments where the protected plants (AGI) were exposed to field inoculation.

~ No. of plants showed fruit damage.

Inoculation by aphids.